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13. ABSTRACT *(Maximum 200 words)*

The development and progression of cancer result from multiple genetic changes accumulated in the cells. The identification of tumor suppressor genes inactivated and proto-oncogenes activated in mammary epithelial cells is essential to understand the genetic basis of breast cancer and is a prerequisite for development of strategies for prevention, diagnosis, and treatment. In breast cancer, loss of heterozygosity (LOH) was detected frequently on chromosome 17 and other chromosomes, suggesting unrecognized tumor suppressor genes. We are applying the novel retroviral-tagging strategy to identify the genes using chromosome 17-suppressed (independent of p53 and BRCA1) breast cancer cell lines. In contrast to the parental tumorigenic cell line CAL51, the suppressed sublines CAL/17-3 and CAL/17-5 display retard growth in flasks, no growth in soft-agar culture and athymic nude mice. In this annual report, we present preparation of biological materials including cell culture, isolation of DNA and RNA, construction of cDNA library and packaging retrovirus particles. In order to provide antisense and mutant proteins to inhibit activities of tumor suppressor genes, poly-(A)$^{+}$RNA was isolated from the suppressed subline CAL17-3 and used to construct the library. We are now in the process to package cDNA library into retrovirus particles for transduction.

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FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

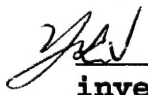_____ Where copyrighted material is quoted, permission has been obtained to use such material.

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INTRODUCTION

Breast cancer is the most common malignancy in Western women, affecting up to one in 10 women during their lifetime. The large majority (approximately 90%) of breast carcinomas is sporadic despite of identification of *BRCA1* and *BRCA2* that are known to involve in familial cases. The development of breast cancer is believed to result from multiple genetic changes accumulating in mammary epithelial cells. In searching for the genetic changes, increasing interest has been focused on tumor suppressor genes involved in control of normal mammary epithelial cells from tumorigenic transformation.

While many investigators have observed LOH in breast cancers on chromosomes 1p, 1q, 3p, 6q, 7q, 11p, 13q, 16q, 17p, 17q, and 18q at the high frequencies variable from 20-60%, chromosome 17 is one of the most frequent carriers of LOH (1-19). On 17p, two distinct regions, 17p13.1 (containing *p53*) and 17p13.3, have shown LOH with frequencies ranging from 30-60% and 60-70%, respectively (10-11, 20-26). On 17q, three regions of frequent LOH have been identified. 17q21 LOH (13, 26-30) contains *BRCA1* (31). LOH at 17q11.1-q12 was detected as frequently as 79% in sporadic breast cancer (32). The third LOH is telomeric to *BRCA1* (26, 29). These studies demonstrate that, in addition to *p53*, *BRCA1, BRCA2*, and others, chromosome 17 and other chromosomes with the high frequent areas of LOH harbor unrecognized tumor suppressor genes involved in the control of the normal growth of mammary epithelial cells.

Direct evidence supporting the existence of additional breast cancer suppressor genes comes from introduction of a *neo*-tagged chromosome 17 into breast cancer cell lines by microcell-mediated chromosome transfer that demonstrated suppression of tumorigenicity (33-37). Casey *et al.* was the first to provide biological evidence that *in vitro* growth of the breast cancer cell line MCF 7 (carrying wild-type *p53*) was suppressed by the introduction of a *neo*-tagged chromosome 17 (33). A very similar result was independently reported by Negrini *et al.* (34). In addition, anchorage-independent growth, cell growth rate on plastic plates, and tumorigenicity in athymic nude mice of the mammary carcinoma cell line R30 were suppressed by introduction of chromosome 17. Wild-type *p53* was not involved in this suppression (35). Furthermore, only the long arm of the transferred chromosome 17 was capable of suppressing the tumorigenicity of the *p53*-mutant breast cancer cell line MDA-MD-231 (34). Finally, Theile *et al.* demonstrated that suppression of tumorigenicity of the breast cancer cell line CAL51 by an introduced chromosome 17 did not require transfer of *p53* or *BRCA1* (37). Thus, additional tumor suppressor genes on chromosome 17 have yet to be identified.

To facilitate identification of tumor suppressor genes we have developed a novel strategy to reverse monochromosome-mediated tumor suppression by retroviral insertional mutagenesis and/or functional inactivation mediated by expressed cDNA fragments (38). Insertional mutagenesis disrupts tumor suppressor genes (e.g., *APC* for familial adenomatous polyposis [39] and *p53* in osteosarcoma [40]) and has been used as a powerful tool to identify various genes including *Fli-1, p53, erb-B*, and *myc* (41-43), *vin-1/cyclin D2* (44), *Tiam-1* (45), *bcar-1*, and *CRL-1* (46-47). Furthermore, it is known that retroviral insertion could activate proto-oncogene. Finally, functional inactivation mediated by expressed cDNA fragments has been achieved by knockout of gene function

(48) and by methods designed to identify genetic suppressor elements by antisense cDNA or dominant negative mutant proteins (49-51). Combining all components of these established approaches into a single system of retroviral-vector-cDNA, we have developed a novel strategy for identification of genetic loci and tumor suppressor genes (38).

"Suppression and reversion of suppression" are the two basic aspects of this novel strategy. "Suppression" refers to the suppression of tumorigenic phenotypic features including anchorage-independent growth, focus formation in plastic culture, rapid cell population doubling time, and tumor formation in athymic nude mice by introduction of a *neo*-tagged monochromosome into a cancer cell line via a microcell mediated chromosome transfer. "Reversion of suppression" means the reversion to the tumorigenic phenotype induced by insertional mutagenesis (proviral tagging) and/or functional inactivation of the suppressor gene(s) by antisense or dominant negative mutant proteins following the transduction of a retrovirus expression vector-carried cDNA library into the monochromosome suppressed cells.

The hypothesis underlying this approach is the following. (1) The phenotypic reversion can derive from inactivation of tumor suppressor genes. The suppression related genetic locus or loci on the introduced chromosome provide targets to insertional mutagenesis. In addition, derived from cancer cell lines, the suppressed sublines may carry many mutated genetic loci that leave functional counterparts being "haploid" targets to insertional mutagenesis. Furthermore, the suppressor gene products can be targets to functional inactivation by antisense and mutant proteins. The poly(A)$^+$-RNA from the suppressed cancer cell line is used to construct cDNA library and then re-introduced to the suppressed cell line to induce the reversion, reasoning that cDNA fragments of the suppressor genes would be present in the library providing antisense and mutant proteins to inhibit activities of the suppressor genes. It is well known that the phenotypic reversion can also come from activation of proto-oncogenes. (2) The successfully transduced tumorigenic cells can be positively selected in soft agar culture following co-selection for the drug-resistance genes on both the suppressive chromosome and the retroviral vector. (3) The cDNA and genomic sequences tagged to the vectors and involved in the tumorigenic reversion can be readily isolated by PCR-based techniques. Based on this hypothesis we have successfully generated a serial retroviral-tagged revertant cell sublines identified important genetic loci (38) and possibly a tumor suppressor gene (unpublished data), using the chromosome-6 suppressed melanoma cell line UACC903(+6).

We are now applying the same strategy to identify unrecognized tumor suppressor gene(s) from a chromosome-17 suppressed breast cancer cell sublines. This study is using the tumorigenic cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3, and CAL17-5 (37). The parental CAL51 cell line demonstrates rapid population doubling time, focus formation in plastic culture, anchorage-independent growth, and rapid formation of subcutaneous tumors in athymic nude mice. All four of these readily detectable phenotypic features are suppressed in the chromosome 17 containing cell sublines (37). The central goal of this project is to identify breast cancer suppressor genes. The specific aims include (1) use of the chromosome-17 suppressed breast cancer cell sublines CAL/17-1, CAL17-3 and CAL17-5 (37) to generate the anchorage-independent revertants by transduction of a retroviral expression vector-carried

cDNA library and (2) use of the anchorage-independent revertants to identify previously unrecognized suppressor genes in breast cancer. Table 1 is the timetable from our original proposal.

Table 1.   The Timetable for the Proposed Experiments

Specific Aim 1	Specific Aim 2
Year 01	
- Completion of the library construction	
- Completion of the retrovirus package	
- Completion of the retrovirus transduction	
Year 02	
- Completion of soft agar selection for colony forming cells and establishment of revertant sublines	
	- Study of *in vitro* growth and tumorigenicity test
	- Identification of the effective cDNAs
Year 03	
	- Mapping of identified cDNAs to chromosome regions
	- Characterization of the full-length cDNAs

BODY

MATERIALS, METHODS, AND PROCEDURES

Cell Culture. The parental breast cancer cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3, and CAL/17-5 (37) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 ug/mL streptomycin sulfate. Six hundred ug/mL of G418 were added in culture of the chromosome 17-containing cells to select for the introduced human normal chromosome 17 tagged with *psv2neo*. NIH3T3, Bosc23 cells (52) and Bing (63) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 ug/mL streptomycin sulfate. Ten% of newborn calf serum was used for culture of GP+envAM12 cells (54). Eight mM of L-histidinol dihydrochloride (hisD^R) was used for cells containing retrovirus vectors. All media, serum, and antibiotics were from Gibco BRL with exceptions where indicated. *Escherichia coli* strain DH5α cells (GIBCO, BRL) containing plasmid pLM2 (38) and strain Supercompetent cells (Catalogue no. 230140; Stratagene) containing a cDNA library were cultured in Luria-Bertani medium with 100 ug of ampicillin per ml.

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Techniques of the Molecular Cloning. Genomic DNA and total RNA were isolated by standard methods (55). Poly(A)$^+$-RNA was extracted from total RNA using the FastTrack 2.0 mRNA Isolation Kit according to manufacturer's instructions (Catalog nos. K1593-02, K1593-03; Invitrogen). The cDNA library was synthesized from poly(A)$^+$ RNA using Universal RiboClone cDNA Synthesis System (Catalog no. C4360; Promega) according to the manufacturer's instruction. Briefly, first strand synthesis was driven by Avian Myeloblstosis Virus (AMV) reverse transcriptase and random hexameric primers, followed directly by second strand replacement synthesis using Rnase H and DNA polymerase I. After treatment with T4 DNA polymerase to flush the ends, the double-stranded cDNA molecules were prepared for cloning by size fractionation and the additino of *Eco*RI adaptors. The resulting cDNA samples were cloned into pLM2 plasmid vectors. Plasmid DNA was isolated using alkaline lysis methods (56).

Packaging of Retrovirus Particles. Virus particles containing retroviral vectors were packaged from pLM2 plasmids, using the retrovirus-packaging cell lines Bosc-23 (ecotropic) and GP+envAM12 (amphotropic) by methods essentially as described (52, 54). Briefly, 20 ug of plasmid DNA was used to transfect approximately 1×10^6 Bosc-23 cells by Ca^{++} coprecipitation using the CellPhect Transfection Kit (Pharmacia Biotech). Serial 10-fold dilution of the supernatant from the transfected cells was used to transduce NIH3T3 cells for the determination of an initial titer of retrovirus particles by histidinol resistance [8 mM L-histidinol dihydrochloride (Sigma Chemical Co.)]. The titer of the packaged retrovirus was approximately 1×10^5 colony forming units per ml. To generate a high titer of amphotropic retrovirus particles, 10 ml of supernatant from transfected Bosc-23 cells were used to transduce aliquots of 1×10^6 GP+envAM12 cells in the presence of 6 ug Polybrene per ml (54). Transduced GP+envAM12 cells were selected for with L-histidinol. A serial 10-fold dilution of supernatant from *his*^{R} cells was exposed to NIH3T3 cells to determine the titer of amphotropic retrovirus particles (1×10^6). The method to package retrovirus particles using Bing cell line (52, 53) was the same as above.

RESULTS AND ASSUMPTIONS

Isolation of genomic DNA, total RNA, and poly(A)$^+$RNA from the parental breast cancer cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3 and CAL/17-5. The genomic DNA have been isolated from the cancer cells and the suppressed cells for the future Southern analysis of restriction fragment length polymorphism in comparison to those of the revertant cell sublines to be generated. Total RNA have been isolated from three cell lines including CAL51, CAL/17-3, and CAL/17-5 for the future Northern analysis in comparison to RNA from the revertant cell sublines to be generated. Poly(A)+RNA was isolated from the chromosome 17 suppressed cell subline CAL/17-3 for construction of the cDNA library described below. We failed to isolate both genomic DNA and total RNA from the suppressed cell subline CAL/17-1.

Construction of random-primed and orientation-independent cDNA library in retroviral expression vector pLM2. Poly(A)$^+$-RNA was isolated from the chromosome 17 suppressed cell subline CAL/17-3 and used to synthesize the cDNA library. The reason for using the suppressed cell subline as the RNA source is that the gene(s) involved in the chromosome-17 mediated tumor suppression would be present in the RNA. Thus, the resulting cDNA library could provide possible antisense fragments and mutant proteins for inhibition of activities of suppressor gene products associated to the chromosome-17 mediated suppression.

4 ug poly(A)$^+$-RNA from CAL/17-3 cell line was used to synthesize the cDNA library. Figure 1 represents the synthesized cDNA samples and controls (1.2 kb kanamycin poly(A)$^+$-RNA). The majority of the double-stranded cDNA fragments range from 0.3 kb to 1.5 kb. After removing the fragments less than 0.4 kb by size fractionation and addition of *Eco*RI adapters, 0.5 ug cDNA preparations were cloned into *Eco*RI site of pLM2 vectors. This library will be used for both packaging retrovirus particles and isolation of cDNA clones after identification of the retroviral tagged and reversion-related sequences.

pLM2 is the plasmid vector carrying hisD^R retroviral expression vector. Upon introduction into human cells, the cDNA inserts in the retroviral expression vector can be transcribed from the CMV promoter-enhancer and the sense-oriented cDNA can also be translated from the introduced translational start codon (ATG) and to a stop codon (43). The stop codon can be from the cDNA inserts or on the vector (TGA) in all three reading frames. Therefore, the expression of possible antisense fragments or mutant proteins in the chromosome 17 suppressed cell sublines is expected to knockout activities of the suppressor gene products.
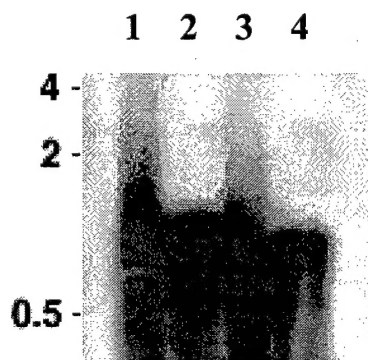
Figure 1. The radioactive labeled cDNA library and controls demonstrate the size ranges of the library synthesized from the CAL/17-3 mRNA. Lanes: 1. A sample of the first strand cDNA library; 2. The first strand cDNA control synthesized from 1.2 kb kanamycin mRNA; 3. A sample of the double stranded cDNA library; 4. The double stranded cDNA control synthesized from 1.2 kb kanamycin mRNA. The sizes of the molecular markers are indicated in the left of the picture in kilobases.

Packaging of Retrovirus Particles. The pLM2 vectors was packaged into retrovirus particles using the packaging cell lines BOSC-23 (ecotropic; Ref. 52) and GP+envAM12 (amphotropic; Ref. 54). The initial titer of the retrovirus particles packaged by BOSC-23 cells was approximately 1×10^5 cfu per ml determined by the number of NIH3T3 colonies resistant to L-histidinol. Aliquots of 10 ml of supernatant (1×10^6 cfu) were used to transduce aliquots of 1×10^6 GP+envAM12 cells to produce supernatant containing a high titer of amphotropic retrovirus particles (1×10^6 cfu/ml) to transduce human cells. These particles are for control experiments. We have also packaged retrovirus particles using Bing cell line. Bing cell line is the amphotropic counterpart to the Bosc23 ecotropic envelop-expression packaging line (52, 53).

DISCUSSION AND RECOMMENDATIONS

In the initial research year, we have successfully prepared biological materials for the downstream work although we moved the laboratory from the National Human Genome Research Institute at the National Institutes of Health to Institute of Molecular and Human Genetics and Lombardi Cancer Center at Georgetown University Medical Center. The genomic DNA and total RNA were isolated from the parental breast cancer cell line CAL51 and the chromosome 17 suppressed cell sublines CAL/17-3 and CAL/17-5. The poly(A)+-RNA isolated from CAL/17-3 was used to synthesize cDNA library that was constructed into the retroviral expression vector pLM2. We have packaged retrovirus particles from the pLM2 plasmid vectors. The retrovirus particles without cDNA inserts will be used as the controls for the tranduction experiments. Using amphotropic retrovirus packaging cell line Bing to package the particles reduces the two-step packaging (first in Bosc23 cell line and then in GP+envAM12 cell line) into one-step (only in Bing cell line) for saving both time and money without detrimental to our research.

In our original proposal, we planed to construct a random-primed and normalized cDNA library with 200-600 bp inserts. We constructed the random-primed cDNA library and omitted the normalization step. One reason is that we can narrow down to the candidate tumor suppressor genes without the normalization. This view came out from our identification of tumor suppressor gene(s) using the chromosome 6 suppressed melanoma cell line UACC903(+6) and the retroviral transduced tumorigenic revertant cell subline SRS3 (unpublished data). In addition, a total of $298,962 was awarded for a 3-year period to the NIH. Because this grant was originally made to a federal facility no indirect costs were included in the award. After moving from the National Institutes of Health to Georgetown University Medical Center the funding level for this project was cut off 20% for the institution indirect cost. Thus, it is necessary for us to omit few experiments to make sure that the overall project succeeds.

The progress of our research in the first year was not as fast as we would like to. The delay was mainly due to moving and setting up the laboratory, recruiting and training researchers. For this very reason, I have requested no cost extension for this award (please see a copy of the letter in Appendix). In addition, we had a difficult time to culture the chromosome 17 suppressed cell subline CAL/17-1. CAL/17-1 was the cell

subline we had proposed to use in the project. However, in our hand, the CAL/17-1 cell line was nearly no dividing in the Petri-dishes after thawing from the liquid nitrogen storage culture. After repeated failure to culture CAL/17-1 cell subline, we changed to use both CAL/17-3 and CAL/17-5 cell sublines. These two sublines turned out to be healthy and their growth is significantly slower than the parental tumorigenic cell line CAL51. We do not know what caused the CAL/17-1 cell line to stop growing. We know CAL/17-1 was a clone from the 8th passage after introduction of chromosome 17, CAL/17-3 from the 6th passage, and CAL/17-5 from the 10th passage (personnel communication with Dr. Micheal Theile). This difference should not be the reason for the problem. We simply changed the cell lines and moved on because the selection for tumorigenic revertant cells will be conducted in soft-agar culture, where CAL/17-3 and CAL/17-5 like CAL/17-1 do not grow (they are anchorage-dependent) and can be used instead.

CONCLUSIONS

In conclusion, we have successfully prepared biological materials including:

(1)   Isolation of genomic DNA and total RNA from the parental breast cancer cell line CAL51 and the chromosome 17 suppressed cell sublines CAL/17-3 and CAL/17-5.

(2)   Isolation of poly(A)+-RNA from CAL/17-3, synthesis and construction of cDNA library into the retroviral expression vector pLM2 for current packaging of retrovirus particles and later identification of the retroviral tagged and reversion-related sequences.

(3)   Packaging of retrovirus particles from the pLM2 plasmid vectors for the coming transduction experiments.

REFERENCES

1. Genuardi M, Tsihira H, Anderson DE, Saunders GF. (1989) Distal deletion of chromosome 1p in ductal carcinoma of the breast. Am J Hum Genet 45: 73-82.

2. Chen L-C, Dollbaum C, Smith HS. (1989) Loss of heterozygosity on chromosome 1 in human breast cancer. Proc Natl Acad Sci USA 86: 7204-7.

3. Gendle, SJ, Cohen EP, Craston A, Duhig T, Johnstone G, Barnes D. (1990) The locus of the polymorphic epithelial mucin (PEM) tumour antigen on chromosome 1q21 shows a high frequency of alteration in primary human breast tumours. Int J Cancer 45: 431-5.

4. Devilee P, Van den Broek M, Kuipers-Dijkshoorn N, Kolluri R, Rhan PM, Cornelisse CJ. (1989) At least four different chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. Genomics 5: 554-60.

5. Devilee P, van Vliet M, van Sloun P, Dijkshoorn NK, Hermans J, Pearson PL, Cornelisse CJ. (1991) Allelotype of human breast carcinoma: a second major site for loss of heterozygosity is on chromosome 6q. Oncogene 6: 1705-11.

6. Bieche J, Champeme MH, Matifas F, Hacene K, Callahan R, Lidereau R. (1992) Loss of heterozygosity on chromosome 7q and aggressive primery breast cancer. Lancet 339: 139-43.

7. Ali IU, Lidereau R, Theillet C, Callahan R. (1987) Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science (Washington DC) 238: 185-8.

8. Lundberg C, Skoog L, Cavenee WK, Nordenskjold M. (1987) Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc Natl Acad Sci USA 84: 2372-6.

9. Radford DM, Fair KL, Phillips NJ, Ritter JH, Steinbrueck T, Holt MS, Donis-Keller H. (1995) Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p,13q, 16q, 17p and 17q. Cancer Res 55: 3399-405.

10. MacKey J, Steel CM, Elder PA, Forrest APM, Evens H-J. (1988) Allele loss on the short arm of chromosome 17 in breast cancers. Lancet 2: 1384-5.

11. Coles C, Thompson AM, Elder PA, Cohen BB, MacKenzie, Cranston G, Chetty U., MacKay J, Macdonald M, NakamuraY, Hoyheim B, Steel CM. (1990) Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. Lancet 336: 761-3.

12. Hall JM, Lee MK, Newman B, Morrow JE, Anderson LA, Huey B, King MC. (1990) Linkage of early-onset familial breast cancer to chromosome 17q21. Science (Washington DC) 250: 1684-9.

13. Narod SA, Feunteun J, Lynch HT, Watson P, Conway T, Lynch J, Lenoir GM. (1991) Familial breast-overian cancer on chromosome 17q12-q23. Lancet 338: 82-3.

14. Devilee P, van Vliet M, Kuipers-Dijkshoorn N, Pearson PL, Cornelisse CJ. (1991) Somatic genetic changes on chromosome 18 in breast cancer: is the DCC gene involved? Oncogene 6: 311-5.

15. Dutrillaux B, Gerbault-Seureau M, Zafrani B. (1990) Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 paradiploid cases with few chromosome changes. Cancer Genet Cytogenet 49: 203-17.

16. Mitchell EL, Santibanez-Koref MF. (1990) 1p13 is the most frequently involved band in structural rearrangements in human cancer. Genes Chromosomes Cancer 2: 278-89.

17. Hainsworth PJ, Raphael KL, Stillwell RG, Bennet RC, Garson OM. (1991) Cytogenetic features of twenty-six primary breast cancers. Cancer Genet Cytogenet 53: 205-18.

18. Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y. (1991) Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res 51: 5794-9.

19. Pandis N, Helm S, Bardi G, Idvall I, Mandahl N, Mitelman F. (1993) Chromosome analysis of 20 breast carcinomas: cytogenetic muticlonality and karyotypicpathologic correlations. Genes Chromosomes Cancer 6: 51-7.

20. Chen LC, Neubauer A, Kurisu W, et al. (1991) Loss of heterozygosity on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. Proc Natl Acad Sci USA 88: 3847-51.

21. Deng G, Chen LC, Schott DR, Thor A, Bhargava V, Ljung BM, Chew K, Smith HS. (1994) Loss of heterozygosity and *p53* gene mutations in breast cancer. Cancer Res 54: 499-505.

22. Cornelis RS, van Vliet M, Vos CB, Cleton-Jansen AM, van de Vijver MJ, Peterse JL, Khan PM, Borresen AL, Cornelisse CJ, Devilee P. (1994) Evidence for a gene on 17p13.3, distal to TP53, as a target for allele loss in breast tumors without *p53* mutations. Cancer Res 54: 4200-6

23. Sato T, et al. (1990) Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 50: 7184-9.

24. Andersen TI, Gaustad A, Ottestad L, Farrants GW, Nesland JM, Tveit KM, Barresen AL. (1992) Genetic alterations of the tumor suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas. Genes Chrom Cancer 4: 113-21.

25. Isomura M, Tanigami A, Saito H, Harada Y, Katagiri T, Inazawa J, Ledbetter DH, et al. (1994) Detailed analysis of loss of heterozygosity on chromosome band 17p13 in breast carcinoma on the basis of a high-resolution physical map with 29 markers. Genes Chrom Cancer 9: 173-9.

26. Kirchweger R, Zeillinger R, Schneeberger C, Speiser P, Louason G, Theillet C. (1994) Patterns of allele losses suggest the existence of five distinct regions of LOH on chromosome 17 in breast cancer. Int J Cancer 56: 193-9.

27. Hall JM, et al. (1992) Closing in on a breast cancer gene on chromosome 17q. Am J Hum Genet 50: 1235-42.

28. Cornelis RS, Devilee P, van Vliet M, Kuipers-Dijkhoorn N, Kersenmaeker A, Bardoel A, Khan PM, Cornelisse CJ. (1993) Allele loss patterns on chromosome 17q in 109 breast carcinomas indicate at least two distinct target regions. Oncogene 8: 781-5.

29. Cropp CS, Champeme M-H, Liderau R, Callahan R. (1993) Identification of three regions on chromosome 17q in primary human breast carcinomas which are frequently deleted. Cancer Res 53: 5617-9.

30. Borg A, Zhang Q-X, Johannsson O, Olsson H. (1994) High frequency of allelic imbalance at the *BRCA1* region on chromosome 17q in both familial and sporadic ductal breast cancinoma. J Natl Cancer Inst 86: 792-4.

31. Miki Y, Swensen J, Shattuck-Eidens D, et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. Science 266: 66-71.

32. Futreal PA, Soderkvist P, Marks JR, Iglehart JD, Cochran C, Barrett JC, Wiseman RW. (1992) Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymophisms. Cancer Res 52: 2624-7.

33. Casey G, Plummer S, Hoeltge G, Scanlon D, Fasching C, Stanbridge EJ. (1993) Functional evidence for a breast cancer growth suppressor gene on chromosome 17. Human Mol Genet 2: 1921-7.

34. Negrini M, Sabbioni S, Haldar S, Possati, Castagroli A, Corallini A, et al. (1994) Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. Cancer Res 54:1818-24.

35. Theile M, Hartmann S, Naundorf H, Rueb D, Elbe B, Krause H, Deppert W, Barrett JC, Scherneck S. (1994) Wild-type *p53* is not involved in reversion of the tumorigenic phenotype of breast cancer cells after transfer of normal chromosome 17. Int J Oncol 4: 1067-75.

36. Chen P, Ellmore N, Weissman BE. (1994) Functional evidence for a second tumor suppressor gene on human chromosome 17. Mol Cell Biol 14: 534-42.

37. Theile M, Hartmenn S, Scherthan H, Arnold W, Deppert W, Frege R, Glaab F, Haensch W, Scherneck S. (1995) Suppression of tumorigenicity of breast cancer cells by transfer of human chromosome 17 does not require transferred *BRCA1* and *p53* genes. Oncogene 10:439-47.

38. Su YA, Ray ME, Lin T, Bodine DM, Meltzer PS, and Trent JM. (1996) Reversion of monochromosome mediated suppression of tumorigenicity in malignant melanoma by retroviral transduction. Cancer Res 56:3186-3191.

39. Miki Y, Mishisho I, Horii A, Miyoshi Y, Utsunomiya J, Kinzler KW, Vogelstein B, Nakamura Y. (1992) Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. Cancer Res 52: 643-5.

40. Mitreiter K, Schmidt J, Luz A, Atkinson MJ, Hofler H, Erfle V, Strauss PG. (1994) Disruption of the murine *p53* gene by insertion of an endogenous retrovirus-like element (ETn) in a cell line from radiation-induced osteosarcoma. Virology 200: 837-41.

41. van Lohuizen, M. and Berns, A. (1990) Tumorigenesis by slow-transforming retroviruses-an update. Bioch Biophys Acta 1032: 213-35.

42. Peters G. (1990) Oncogenes at viral integration sites. Cell Growth and Differentiation 1: 503-510.

43. Ben-David Y and Bernsterns A. (1991) Friend virus-induced erythroleukemia and the multistage nature of cancer. Cell 66: 831-4.

44. Hanna Z, Jankowski M, Tremblay P, Jiang X, Milatovich A, Francke U, Jolicoeur P. (1993) The Vin-1 gene, identified by provirus insertional mutagenesis, is the cyclin D2. Oncogene 8: 1661-6.

45. Habets GGM, Scholtes EHM, Zuydgeest D, van der Kammen RA, Stam JC, Berns A, and Collard JG. (1994) Identification with an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for rho-like proteins. Cell 77: 537-49.

46. Dorssers LC, van Agthoven T, Dekker A, van Agthoven TL, Kok EM. (1993) Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. Mol Endocrinol 7: 870-8.

47. Lu SJ, Man S, Bani MR, Adachi D, Hawley RG, Kerbel RS, Ben-David Y. (1995) Retroviral insertional mutagenesis as a strategy for the identification of genes associated with cis-diamminedichloroplatinum (II) resistance. Cancer Res 55: 1139-45.

48. Deiss LP, Kimchi A. (1991) A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. Science 252:117-20.

49. Holzmayer TA, Pestov DG, Roninson IB. (1992) Isolation of dominant negative mutants and inhibitory antisense RNA sequences by expression selection of random DNA fragments. Nucleic Acids Res 20: 711-7.

50. Gudkov AV, Zelnick CR, Kazarov AR, Thimmapaya R, Suttle DP, Beck WT, Roninson IB. (1993) Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA. Proc Natl Acad Sci USA 90: 3231-5.

51. Gudkov AV, Kazarov AR, Thimmapaya R, Axenovich SA, Mazo IA, Roninson IB. (1994) Cloning mammalian genes by expression selection of genetic suppressor elements: association of kinesin with drug resistance and cell immortalization. Proc Natl Acad Sci USA 91: 3744-8.

52. Pear WS, Nolan G P, Scott ML, Baltimore D. (1993) Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci USA 90: 8392-6.

53. Thony B, Leimbacher W, Stuhlmann H, Heizmann CW, Blau N. (1996) Retroviral mediated gene transfer of 6-pyruvoyl-tetrahydropterin synthase corrects tetrahydrobiopterin deficiency in fibroblasts from hyperphenylalaninemic patients. Human Gene Therapy 7:1587-93.

54. Markowitz D, Goff S, Bank A. (1988) Construction and use of a safe and efficient amphotropic packaging cell line. Virology 167:400-6.

55. Sambrook J, Fritsch EF, Maniatis, T. *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Plainview, NY) pp. 7.6-7.9, 9.16-9.19, 2.108-2.117, 1989.

56. Engebrecht J, Brent R. Minipreps of Plasmid DNA: Alkaline Lysis Miniprep. *In* Current Protocols in Molecular Biology, Vol. 1, Supplement 15 (F.M. Ausubel, ed.) pp. 1.6.1-1.6.2. Current Protocols: Greene Publishing Associates and John Wiley & Sons, Inc., 1991.

APPENDICES

Attached please see the copies of documents regarding:

(1)   No cost extension of the grant number DAMD17-97-1-7236.

(2)   Grant Transfer from NIH.

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September 14, 1998

Patricia Modrow, Ph.D.
Office of the Deputy Chief of
    Staff for Information Management
US Army Medical Research and Materiel Command
Department of the Army
504 Scott Street
Fort Detrick, Maryland 21702-5012

RE: No Cost Extension of Grant Number DAMD17-97-1-7236

Dear Dr. Modrow,

I am writing to request for one-year no-cost extension of the grant number DAMD17-97-1-7236, titled "Identification of Tumor Suppressor Genes in Breast Cancer by Insertional Mutagenesis and Functional Inactivation". I am requesting the extension for the following reason. In the last year, I became a faculty (Assistant Professor) in the Institute for Molecular and Human Genetics, and Lombardi Cancer Center (LCC) at Georgetown University Medical Center (GUMC). I moved to GUMC from the National Human Genome Research Institute (NHGRI) on August 1, 1997. After the move, the grant was successfully transferred from NHGRI to LCC due to cooperative efforts among your organization, NHGRI, and LCC. However, because of establishment and furnishing of the laboratory, recruitment and significant training of personnel, and advancement of research technology, our research has been delayed. Thus, I am requesting one-year no-cost extension of the grant to ensure the completion of the research project.

We are greatly honored by receiving supports for our research from organizations like yours. The Institute for Molecular and Human Genetics, Lombardi Cancer Center, and the NHGRI provide us with direct and collaborative supports, resulting in our several scientific presentations in the State and the International conferences. For an example, I was given a chance to orally present our research during Symposium on Cancer Genetics and Tumor Suppressor Genes at Cold Spring Harbor Laboratory on August 19-23, 1998 and received good response. The funding provided by your organization will tremendously accelerate our breast cancer research.

NCI
CCCC

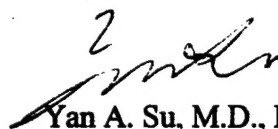Comprehensive Cancer Center Designated by the National Cancer Institute

It is worth mentioning that in the last fiscal year we spent > 6 months to set up the laboratory and recruit new researchers. We are working in an area of advanced cancer genetic fields. It took significant time to train my crew fitting in the advanced technologies. Fortunately, they all work very hard. We are now focusing on carrying out experiments. In short, we are devoted to, competent for, and working hard at fighting against human cancers.

Again, the funding (DAMD17-97-1-7236) from your organization is essential and adds the effective strength to this laboratory to work toward understanding, preventing, and eradicating human breast cancers. In order to ensure the completion of the research project, I am requesting one-year no-cost extension of the funding (total cost of $298,962 for the 3-year period beginning August 25, 1997) from August 25, 1997 – August 24, 2000 to August 25, 1998 – August 24, 2001.

If you have any questions, please feel free to contact me at phone number: 202-687-6056.

I deeply appreciate your support.

Sincerely yours,

Yan A. Su, M.D., Ph.D.
Assistant Professor
Institute for Molecular and Human Genetics
Lombardi Cancer Center
Georgetown University Medical Center

Cc: Ms. Judy Pawlus
            Office of the Deputy Chief of
                Staff for Information Management
            USAMRMC

        Dr. Jeffrey Trent, Ph.D.
            Director, NHGRI
            NIH

        Dr. Marc Lippman, M.D.
            Director, IMHG & LCC
        Ms. Karen Huff, M.S.
            Research Administrator, IMHG & LCC
        Mr. Lenny Fraser,
            Grants and Contracts Officer
            GUMC

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MEMORANDUM

Offices in
Washington, DC
Rockville, MD
Arlington, VA

TO: Greg Raymond
           Acting Chief Financial Officer

THROUGH: Arthur Raines, Ph.D.
           Acting Associate Dean for Research Operations

FROM: Marc E. Lippman, M.D.
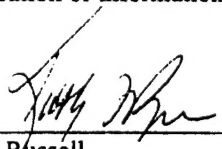   Director

DATE: November 5, 1997

SUBJECT: Grant Transfer From NIH

Dr. Yan Su joined the Lombardi Cancer Center and the Institute for Molecular and Human Genetics in August of this year and now we are in the process of transferring his U.S. Army grant from his previous employer, the National Center for Human Genome Research at NIH. A total of $298,962 was awarded for a 3-year period to the NIH. Because this grant was originally made to a federal facility no indirect costs were included in the award. Therefore, the purpose of this memo is to respectfully request a reduced indirect cost rate for this grant.

We realize that in our present financial climate it would be irresponsible to request a waiver of indirect costs. We are seeking a reduced indirect cost rate of 20% because any indirect costs charged to the grant would reduce the resources available for the research project. Utilizing an indirect cost rate of 20%, the first year funding of $112,000 would be reduced to $89,600 of available funding. We feel anything higher would be severely detrimental to the research Dr. Su has committed to conduct.

Please respond to Lennox Fraser, the Grants and Contracts officer responsible for the transfer of this grant. If further clarification or information is required please contact Karen Huff at 687-3039.

Kathy Russell
Associate Director for
  Planning and Administration
Lombardi Internal Budget Review

MEL/kh
cc: Lennox Fraser
     Yan Su, Ph.D.
     Karen Huff
     Susan Myers

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